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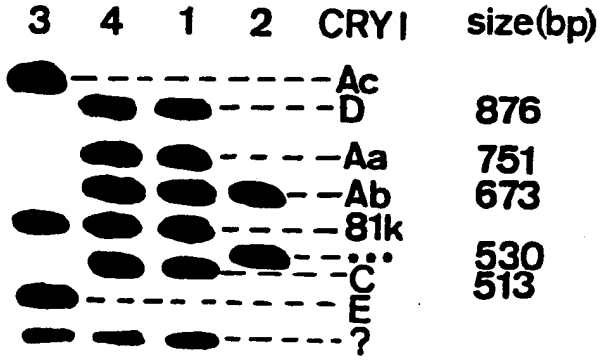
(54) Title: NEW *BACILLUS THURINGIENSIS* STRAINS AND THEIR INSECTICIDAL PROTEINS

(57) Abstract

Four novel *Bacillus thuringiensis* strains, which are deposited at the BCCM-LMG under accession nos. LMG P-12592, LMG P-12593, LMG P-12594, and LMG P-13493, produce new crystal proteins during sporulation that are toxic to Lepidoptera, more particularly against Noctuidae such as *Spodoptera* spp. and *Agrotis* spp., against Pyralidae such as *Ostrinia nubilalis*, against Gelechiidae such as *Phthorimaea operculella*, and against Yponomeutidae such as *Plutella xylostella*, and that are encoded by a novel gene. The crystal proteins contain protoxins, which can yield a toxin as trypsin-digestion product. A plant, the genome of which is transformed with a DNA sequence that comes from either one of the strains and that encodes its respective toxin, is resistant to Lepidoptera. Each strain, itself, or its crystals, crystal proteins, protoxin or toxin can be used as the active ingredient in an insecticidal composition for combatting Lepidoptera.



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NEW BACILLUS THURINGIENSIS STRAINS AND THEIR INSECTICIDAL PROTEINS

This invention relates to four novel strains of Bacillus thuringiensis (the "BTS02617A strain", the "BTS02618A strain", the "BTS02654B strain" and the "BTS02652E strain"), each of which produces crystallized proteins (the "BTS02617A crystal proteins", the "BTS02618A crystal proteins", the "BTS02654B crystal proteins" and the "BTS02652E crystal proteins", respectively) which are packaged in crystals (the "BTS02617A crystals", the "BTS02618A crystals", the "BTS02654B crystals" and the "BTS02652E crystals", respectively) during sporulation. The BTS02617A, BTS02618A, BTS02654B and BTS02652E strains were eposited under the provisions of the Budapest Treaty at the Belgian Coordinated Collections of Microorganisms - Collection Laboratorium voor Microbiologie Belgium ("BCCM-LMG"), R.U.G., K. Ledeganckstraat 35, B-9000 Gent.

This invention also relates to an insecticide composition that is active against Lepidoptera and that comprises the BTS02617A, BTS02618A, BTS02654B or BTS02652E strain, as such, or preferably the BTS02617A, BTS02618A, BTS02654B or BTS02652E crystals, crystal proteins or the active component(s) thereof as an active ingredient.

This invention further relates to a gene (the "bTS02618A gene"), which is present in the genome of the BTS02617A, BTS02618A, BTS02654B and BTS02652E strains and which encodes an insecticidal protein (the "BTS02618A protoxin") that is found in the BTS02617A, BTS02618A, BTS02654B and BTS02652E crystals. The BTS02618A protoxin is the protein that is produced by the BTS02617A, BTS02618A, BTS02654B and BTS02652E strains before being packaged into their respective BTS02617A, BTS02618A, BTS02654B and BTS02652E crystals.

This invention still further relates to a toxin (the "BTS02618A toxin") which can be obtained (e.g., by trypsin digestion) from the BTS02618A protoxin. The BTS02618A toxin

is an insecticidally active protein which can be liberated from the BTS02617A crystals, the BTS02618A crystals, the BTS02654B crystals, and the BTS02652E crystals, which are produced by the BTS02617A strain, the BTS02618A strain, the BTS02654B strain and the BTS02652E strain, respectively. This toxin and its protoxin have a high activity against a wide range of lepidopteran insects, particularly against Noctuidae, especially against Spodoptera and Agrotis spp., but also against other important lepidopteran insects such as Pyralidae, particularly the European corn borer, Ostrinia nubilalis, Gelechiidae such as Phthorimaea operculella and Yponomeutidae such as Plutella xylostella. Furthermore, the BTS02618A protein is the first Bt protein with significant activity towards Agrotis segetum. This new characteristic of the BTS02618A protoxin and toxin ("(pro)toxin"), i.e., the combination of activity against different economically important Lepidopteran insect families such as Noctuidae, Yponomeutidae, Gelechiidae and Pyralidae, makes this (pro)toxin an ideally suited compound for combatting a wide range of insect pests by contacting these insects with the (pro)toxin, e.g., by spraying or by expressing the bTS02618A gene in plant-associated bacteria or in plants. The BTS02618A toxin is believed to represent the smallest portion of the BTS02618A protoxin which is insecticidally effective against Lepidoptera.

This invention also relates to transformed Bacillus thuringiensis strains, containing DNA sequences encoding a BTS02618A protein or variants thereof having substantially the same insecticidal activity.

This invention yet further relates to a chimeric gene that can be used to transform a plant cell and that contains the following operably linked DNA fragments:

1) a part of the bTS02618A gene (the "insecticidally effective bTS02618A gene part") encoding an insecticidally effective portion of the BTS02618A protoxin, preferably a truncated part of the bTS02618A gene (the "truncated bTS02618A gene") encoding just the BTS02618A toxin;

2) a promoter suitable for transcription of the

insecticidally effective bTS02618A gene part in a plant cell; and

3) suitable 3' end transcript formation and polyadenylation signals for expressing the insecticidally effective bTS02618A gene part in a plant cell.

This chimeric gene is hereinafter generally referred to as the "bTS02618A chimeric gene".

This invention also relates to:

1) a cell (the "transformed plant cell") of a plant, such as corn or cotton, the genome of which is transformed with the insecticidally effective bTS02618A gene part, preferably the bTS02618A chimeric gene; and

2) a plant (the "transformed plant") which is regenerated from the transformed plant cell or is produced from the so-regenerated plant and their seeds, the genome of which contains the insecticidally effective bTS02618A gene part, preferably the bTS02618A chimeric gene, and which is resistant to Lepidoptera.

This invention still further relates to :

1) a microbial organism, such as B. thuringiensis or Pseudomonas spp., the genome of which is transformed with all or part of the bTS02618A gene; and

2) a microbial spore, containing a genome which is transformed with all or parts of the bTS02618A gene.

Another embodiment of the present invention relates to artificially made bTS02618A genes which encode BTS02618A proteins, and to proteins which are more protease resistant than native Bt proteins, more preferably the native BTS02618A protein. A particular example of a protein that is more protease resistant is the BTS02618Aa protein. Furthermore, the present invention also relates to a DNA sequence encoding the BTS02618Aa protein.

Yet another embodiment of the present invention relates to a chimeric gene that can be used to transform a plant cell and that contains:

1) a DNA sequence encoding an insecticidally effective portion of the BTS02618Aa protoxin, preferably a truncated

part of the bTS02618Aa gene (the "truncated bTS02618Aa gene") encoding just the BTS02618Aa toxin;

2) a promoter suitable for transcription of the insecticidally effective bTS02618Aa gene part in a plant cell; and

3) suitable 3' end transcript formation and polyadenylation signals for expressing the insecticidally effective bTS02618Aa gene part in a plant cell.

This chimeric gene is hereinafter generally referred to as the "bTS02618Aa chimeric gene".

This invention further relates to:

1) a cell (the "transformed plant cell") of a plant, such as corn or cotton, the genome of which is transformed with the insecticidally effective bTS02618Aa gene part, preferably the bTS02618Aa chimeric gene; and

2) a plant (the "transformed plant") which is regenerated from the transformed plant cell or is produced from the so-regenerated plant and their seeds, the genome of which contains the insecticidally effective bTS02618Aa gene part, preferably the bTS02618Aa chimeric gene, and which is resistant to Lepidoptera.

This invention still further relates to :

1) a microbial organism, such as B. thuringiensis or Pseudomonas spp., the genome of which is transformed with all or part of a DNA sequence encoding the BTS02618Aa protein; and

2) a microbial spore, containing a genome which is transformed with all or part of the bTS02618Aa gene.

Yet another embodiment of the present invention relates to insecticidal compositions that are active against Lepidoptera and that comprise a more protease resistant Bt protein, more particularly the BTS02618Aa protein or a variant thereof which has substantially the same insecticidal activity.

Background of the Invention

B. thuringiensis ("Bt") is a Gram-positive bacterium which

produces endogenous crystals upon sporulation. The crystals are composed of proteins which are specifically toxic against insect larvae. These crystal proteins and corresponding genes have been classified based on their structure and insecticidal spectrum (Höfte and Whiteley, 1989). The four major classes are Lepidoptera-specific (cryI), Lepidoptera- and Diptera-specific (cryII), Coleoptera-specific (cryIII), and Diptera-specific (cryIV) genes.

The fact that conventional submerged fermentation techniques can be used to produce Bt spores on a large scale makes Bt bacteria commercially attractive as a source of insecticidal compositions.

Gene fragments from some Bt strains, encoding insecticidal proteins, have heretofore been identified and integrated into plant genomes in order to render the plants insect-resistant. However, obtaining expression of such Bt gene fragments in plants is not a straightforward process. In order to achieve optimal expression of an insecticidal protein in plant cells, it has been found necessary to engineer each Bt gene fragment in a specific way so that it encodes a part of a Bt protoxin that retains substantial toxicity against its target insects (European patent application ("EPA") 86/300,291.1 and 88/402,115.5; U.S. patent application 821,582, filed January 22, 1986).

Summary of the Invention

In accordance with this invention, four novel Bt strains, i.e., the BTS02617A, BTS02618A, BTS02654B and BTS02652E strains, are provided. The BTS02617A, BTS02618A, BTS02654B and BTS02652E crystals and crystal proteins, the BTS02618A protoxin and toxin produced by the strains during sporulation, and insecticidally effective portions of the BTS02618A protoxin, as well as equivalents of these crystals, crystal proteins, protoxin, toxin and insecticidally effective protoxin portions, each possess insecticidal activity and can therefore be formulated into insecticidal compositions against Lepidoptera

in general, and particularly against Noctuidae, such as Agrotis spp. (cutworms such as Agrotis ipsilon and Agrotis segetum), Mamestra spp. (e.g., the cabbage moth, Mamestra brassica) and Spodoptera spp. (armyworms, such as Spodoptera exigua, Spodoptera frugiperda, Spodoptera littoralis and Spodoptera litura), against Pyralidae (e.g., the European corn borer, Ostrinia nubilalis), against Gelechiidae such as Phthorimaea operculella and Yponomeutidae (such as Plutella xylostella) which are major pests of various economically important crops, such as corn, cotton and many vegetables such as Brassicas.

Also in accordance with this invention, a plant cell genome is transformed with the insecticidally effective bTS02618A gene part, preferably the truncated bTS02618A gene, or an equivalent thereof such as a modified, synthetic bTS02618A gene. It is preferred that this transformation be carried out with the bTS02618A chimeric gene. The resulting transformed plant cell can be used to produce transformed plants, seeds of transformed plants and plant cell cultures consisting essentially of the transformed cells. The transformed cells in some or all of the tissues of the transformed plants: 1) contain the insecticidally effective bTS02618A gene part as a stable insert in their genome, and 2) express the insecticidally effective bTS02618A gene part by producing an insecticidally effective portion of its BTS02618A protoxin, preferably its BTS02618A toxin, thereby rendering the plant resistant to Lepidoptera. The transformed plant cells of this invention can also be used to produce, for recovery, such insecticidal Bt proteins.

Further in accordance with this invention, a process is provided for rendering a plant resistant to Lepidoptera by transforming the plant cell genome with the insecticidally effective bTS02618A gene part, preferably the truncated bTS02618A gene, or an equivalent thereof. In this regard, it is preferred that the plant cell be transformed with the bTS02618A chimeric gene.

Yet further in accordance with this invention, there are provided the BTS02618A protoxin, the insecticidally effective

portions of such protoxin and the BTS02618A toxin, as well as functional parts of the BTS02618A toxin, as well as the bTS02618A gene, the insecticidally effective bTS02618A gene part, the truncated bTS02618A gene and the chimeric bTS02618A gene, as well as their equivalents.

Also in accordance with this invention, a DNA sequence, either natural or artificial, encoding the BTS02618A protoxin or insecticidally effective portions thereof, such as the toxin, is provided.

Also in accordance with this invention are provided an insecticidal composition against Lepidoptera, particularly Noctuidae, Pyralidae, Gelechiidae and Yponomeutidae, and a method for controlling Lepidoptera, particularly Noctuidae, Pyralidae, Gelechiidae and Yponomeutidae, with the insecticidal composition, wherein the insecticidal composition comprises the BTS02617A, BTS02618A, BTS02654B or BTS02652E strain, crystals and/or crystal proteins or the BTS02618A protoxin, toxin and/or insecticidally effective protoxin portions or their equivalents.

Also in accordance with this invention, bacteria, particularly E. coli and Bacillus thuringiensis, transformed to express a DNA encoding the BTS02618A protein variant, such as the BTS02618Aa protein or more improved protease resistant Bt proteins are provided.

Furthermore, in accordance with this invention, an artificial DNA sequence encoding the BTS02618A protein, as well as new forms of Bt proteins with improved protease resistance, more particularly the BTS02618Aa or the modified BTS02618A protein are provided, and DNA sequences encoding these new proteins. Further provided are plant cells expressing an artificial DNA sequence encoding the BTS02618A toxin or Bt toxins with improved protease resistance, more preferably the BTS02618Aa toxin.

Also provided is an insecticidal composition, comprising as an active ingredient the BTS02618Aa protein, or a variant thereof with substantially the same insecticidal activity. Also provided is a method to combat Lepidopteran insects by

contacting these insects with Bt proteins having improved protease resistance, more preferably the BTS02618Aa protein or a variant thereof.

More specifically provided are new Bt proteins, preferably Lepidoptera active Bt proteins, having substantially the same insecticidal activity as the native Bt protein, but characterized in their resistance to further proteolytic cleavage of the about 60 to 70 kD toxin form. Such new Bt proteins have inactivated internal protease cleavage sites, so that these proteins have increased stability while retaining substantially the same insecticidal activity. Thus, these new Bt proteins are not readily cleaved into smaller proteolytic fragments which lower their insecticidal activity upon prolonged incubation in the presence of proteases.

Detailed Description of the Invention

The BTS02618A protoxin of this invention can be isolated in a conventional manner from the BTS02617A strain, deposited on July, 2 at the BCCM-LMG under accession number **LMG P-12592**, the BTS02618A strain, deposited on July 2, 1992 at the BCCM-LMG under accession number **LMG P-12593**, the BTS02654B strain, deposited on July 2, 1992 at the BCCM-LMG under accession number **LMG P-12594**, or the BTS02652E strain deposited on March 1, 1993 at the BCCM-LMG under accession number **LMG P-13493**. For example, the BTS02617A, BTS02618A, BTS02654B or BTS02652E crystals can be isolated from sporulated cultures of their respective strain (Mahillon and Delcour, 1984), and then, the BTS02618A protoxin can be isolated from the crystals according to the method of Höfte et al. (1986). The protoxins can be used to prepare monoclonal or polyclonal antibodies specific for the protoxin in a conventional manner (Höfte et al., 1988). The BTS02618A toxin can be obtained by protease (e.g., trypsin) digestion of the BTS02618A protoxin.

The bTS02618A gene can be isolated in a conventional manner. The bTS02618A gene can be identified in the BTS02617A, BTS02618A, BTS02654B or BTS02652E strain, using the procedure

described in U.S. Patent Application 821,582, filed January 22, 1986, and in EPA 86/300,291.1 and 88/402,115.5 (which are incorporated herein by reference). The bTS02618A gene was identified by: digesting total DNA from one of the above strains with restriction enzymes; size fractionating the DNA fragments, so produced, into DNA fractions of 5 to 10 Kb; ligating these fractions to cloning vectors; screening the E. coli, transformed with the cloning vectors, with a DNA probe that was constructed from a region of the cryIG gene (Smulevitch et al., 1991; Gleave et al., 1992).

The term "bTS02618A gene" as used herein includes a DNA sequence encoding the BTS02618A protoxin or toxin or functionally equivalent variants thereof. Indeed, because of the degeneracy of the genetic code, some amino acid codons can be replaced with others without changing the amino acid sequence of the protein. Furthermore, some amino acids can be substituted by other equivalent amino acids without significantly changing the insecticidal activity of the protein. Also, changes in amino acid composition in regions of the molecule, different from those responsible for binding and toxicity are less likely to cause a difference in insecticidal activity of the protein. Such equivalents of the gene include DNA sequences hybridizing to the DNA sequence of the BTS02618A toxin or protoxin of SEQ ID. No. 4 and encoding a protein with the same insecticidal characteristics as the BTS02618A (pro)toxin, of this invention. In this context, the term "hybridization" refers to conventional hybridization conditions, most preferably stringent hybridization conditions.

The "BTS02618A protein" is a general term for the BTS02618A protoxin and variants or mutants thereof with substantially the same insecticidal activity; for example, the BTS02618A or BTS02618Aa toxins.

As used herein, the term "more or improved protease resistant protein" means that the Bt protein fragment resulting from protease cleavage of the native protoxin does not result in a substantial loss of insecticidal activity due to the further cleavage of the insecticidally active toxin part of the

protein. It is preferable that the insecticidally active toxin part of the protein be about 60 to 70 kD and more particularly that the further cleavage is at the N-terminal part of the toxin. It is also preferred that the protein is insecticidal for Lepidoptera.

A preferred example of an "alternative form" of the bTS02618A gene is the artificial bTS02618A gene of SEQ ID. No. 6, encoding a BTS02618A toxin. A further preferred example of an artificial bTS02618A gene is illustrated in the DNA sequence of SEQ ID. No. 8, for reasons of clarity further named "the bTS02618Aa gene", encoding a (similar but different) protein with an insecticidal activity substantially similar to the BTS02618A protein.

Of course, the present invention is not limited to the particular preferred embodiments described herein as "alternative variants or forms." In fact, any other DNA sequences differing in their codon usage but encoding the same protein or a similar protein with substantially the same insecticidal activity, can be constructed by the person skilled in the art. In some prokaryotic and eucaryotic expression systems, for example, changing the codon usage to that of the host cell can increase gene expression (Bennetzen & Hall, 1982; Itakura, 1977). Moreover, since many Bt genes are known to have no bias towards eucaryotic codons, and to have very AT-rich genes it is sometimes beneficial to change the codon usage (Adang et al., 1985, Schnepf et al., 1985). To accomplish this codon usage tables which are available in the literature (Wada et al., 1990; Murray et al., 1989) and in the major DNA sequence databanks (e.g., EMBL at Heidelberg, Germany) are often referred to by the person skilled in the art. Accordingly, synthetic DNA sequences can be constructed so that the same or substantially the same proteins may be produced. See, for example, Cohen et al., 1973.

The term "substantially the same", when referring to a protein, is meant to include a protein that differs in some amino acids, or has some amino acids added (e.g., a fusion protein, see Vaeck et al., 1987) or delet d (e.g., N- or C-

terminal truncation), while retaining the protein's insecticidal activity. It is generally known to those skilled in the art that general amino acid replacements in many parts of a polypeptide chain may be made without seriously modifying the activity of the polypeptide (Watson et al Molecular Biology of the Gene (1987) 226-227.

The term "functional parts of the BTS02618A toxin" as used herein means any part(s) or domain(s) of the toxin with a specific structure that can be transferred to another (Bt) protein for providing a new hybrid protein with at least one functional characteristic (e.g., the binding and/or toxicity characteristics) of the BTS02618A toxin (Ge et al., 1991). Such parts can form an essential feature of the hybrid Bt protein with the binding and/or toxicity characteristics of the BTS02618A protein. Such a hybrid protein can have an enlarged host range, an improved toxicity and/or can be used in a strategy to prevent insect resistance development (European Patent Publication ("EP") 408 403; Visser et al., 1993).

The "BTS02618Aa toxin", as used herein, refers to a new form of the BTS02618A toxin, differing in some amino acids from the native BTS02618A toxin. Indeed, the BTS02618A protoxin has been found to be digested by proteases into an about 69 kD protein and about 55 kD protein, the latter having substantially lower insecticidal activity. The longer the protease digestion, the more of the about 55 kD protein was formed. This 55 kD protein was found to be cleaved by digestion at the Arginine at amino acid position 164 shown in SEQ ID. No. 4. Thus, in the BTS02618Aa toxin, the Arginine at this position was replaced with a Lysine, the 43 N-terminal amino acids was replaced by amino acids Met-Ala, and the C-terminal end was truncated up to amino acid 666 (in SEQ ID. No. 4). Similar to other Bt toxins, the C-terminal end of the BTS02618Aa toxin can be further truncated to the minimum toxic fragment (up to amino acid 658 in SEQ ID. No. 4).

In another form of the BTS02618A protein, "the BTS02618Ab protein", this Arginine has been substituted with an Alanine. Both the BTS02618Aa/b proteins are less susceptible to

proteases and still have substantially the same insecticidal activity. The part C-terminal from the toxic fragment of both the BTS02618Aa and BTS02618Ab protoxins is 100% identical to the C-terminal part of the BTS02618A protoxin.

The "bTS02618Aa gene" and the "bTS02618Ab gene", as used herein, refer to DNA sequences encoding respectively the BTS02618Aa and BTS02618Ab proteins. It is evident that several DNA sequences can be devised once the amino acid sequence of the BTS02618Aa and BTS02618Ab proteins are known. Such other DNA sequences include synthetic or semi-synthetic DNA sequences that have been changed in order to inactivate certain sites in the gene, e.g., by selectively inactivating certain cryptic regulatory or processing elements present in the native sequence as described in PCT publications WO 91/16432 and WO 93/09218, or by adapting the overall codon usage to that of a more related host organism, preferably that of the host organism, in which expression is desired.

Such a modification of the BTS02618A protein can also be achieved by deleting the Arginine at amino acid position 123, or by replacing this amino acid by another amino acid provided that the insecticidal activity of the new BTS02618A protein is not substantially changed. Other amino acids surrounding the protease cleavage site can also be altered such that the insecticidal activity is not substantially changed.

The new proteins can be tested in routine bio-assays to compare their toxicity with that of the native BTS02618A protein. The overall toxicity parameters of such proteins should be similar to those of the native proteins.

Due to the retention of their insecticidal activity, such new proteins are very useful for combatting important pest insects. Their improved resistance for protease activity makes them the toxins of choice for combatting insects, e.g., by expressing a DNA sequence encoding such proteins in a foreign host, such as bacteria or plants. Small modifications to a DNA sequence such as described above are routinely made by PCR-mediated mutagenesis (Ho et al.,1989, White et al., 1989).

The above variants show that indeed modifications can be

made to the BTS02618A protein without causing any substantial changes to the insecticidal activity. Besides a deletion of up to 43 amino acids at the N-terminus, and a major deletion of C-terminal amino acids, also some internally located amino acids can be replaced by others while retaining substantially the same insecticidal activity of the BTS02618A toxin.

Similarly, the CryIB protoxin (Brizzard & Whiteley, 1988), and a naturally occurring variant thereof (EP publication 408 403) has been found to be cleaved into an about 69 kD toxin and a smaller about 55 kD toxin by protease activity. Also for this toxin, modification of the Arginine at amino acid positions 144 and 146 (relative to the start codon) in the sequence of Brizzard & Whiteley (1988) or the sequence of EP 408 403 can increase the stability of the protein in the insect gut. Indeed, prolonged protease treatment of the CryIB protoxin, either obtained from Bt strain 4412 or expressed in E. coli, resulted in an about 55 kD protein with an N-terminal end starting at amino acid position 145 (Thr-Arg-Ser-Val-Leu-) and another about 55 kD protein starting at position 147 (Ser-Val-Leu-Tyr-Thr-). Modifying the Arginine amino acids at positions 144 and 146 leads to a more stable toxin form, which is still toxic. This modification can be incorporated into a natural or synthetic DNA sequence encoding the CryIB protein or variants thereof such as the Bt14 toxin in EP 358 557, by techniques well known in the art, so that a more stable CryIB protein is produced. Such a CryIB protein can be used together with the BTS02618A or BTS02618Aa protein and the CryIAb protein in combatting Lepidopteran insects, particularly Ostrinia nubilalis, by expressing DNA sequences encoding these proteins in a host cell, particularly a plant cell. So the modification of one or more amino acids is useful in other Bt proteins, particularly anti-Lepidoptera Bt proteins, that are also further cleaved by proteases.

Furthermore, the 5 to 10 Kb fragments, prepared from total DNA of the BTS02617A or BTS02618A or BTS02654B or BTS02652E strain, can be ligated in suitable expression v ctors and transformed in E. coli, and the clones can then be screened by

conventional colony immunoprobing methods (French et al., 1986) for expression of the toxin with monoclonal or polyclonal antibodies raised against the BTS02618A toxin.

Also, the 5 to 10 Kb fragments, prepared from total DNA of the BTS02617A or BTS02618A or BTS02654B or BTS02652E strain, can be ligated in suitable Bt shuttle vectors (Lereclus et al., 1992) and transformed in a crystal minus Bt-mutant. The clones are then screened for production of crystals (detected by microscopy) or crystal proteins (detected by SDS-PAGE).

The so-identified bTS02618A gene was sequenced in a conventional manner (Maxam and Gilbert, 1980) to obtain the DNA sequence. Hybridization in Southern blots and sequence comparison indicated that this gene is different from previously described genes encoding protoxins and toxins with activity against Lepidoptera (Höfte and Whiteley, 1989).

An insecticidally effective part of the bTS02618A gene, encoding an insecticidally effective portion of its protoxin, and a truncated part of the gene, encoding just its toxin, can be made in a conventional manner after sequence analysis of the gene. The amino acid sequence of the BTS02618A protoxin and toxin was determined from the DNA sequence of the bTS02618A gene and the truncated bTS02618A gene. By "an insecticidally effective part" or "a part" of the bTS02618A gene is meant a DNA sequence encoding a polypeptide which has fewer amino acids than the BTS02618A protoxin but which is still toxic to Lepidoptera.

In order to express all or an insecticidally effective part of the bTS02618A gene or an equivalent gene in E. coli, in other Bt strains and in plants, suitable restriction sites can be introduced, flanking each gene or gene part. This can be done by site-directed mutagenesis, using well-known procedures (Stanssens et al., 1989; White et al., 1989). In order to obtain improved expression in plants, it may be preferred to modify the codon usage of the bTS02618A gene or insecticidally effective bTS02618A gene part to form an equivalent, modified or artificial gene or gene part in accordance with PCT publications WO 91/16432 and WO 93/09218;

EP 0,358,962 and EP 0,359,472. For obtaining enhanced expression in monocot plants such as corn, a monocot intron also can be added to the bTS02618A chimeric gene, and the DNA sequence of the bTS02618A gene part can be further changed in a translationally neutral manner, to modify possibly inhibiting DNA sequences present in the gene part by means of site-directed intron insertion and/or by introducing changes to the codon usage, e.g., adapting the codon usage to that most preferred by the specific plant (Murray et al., 1989) without changing significantly the encoded amino acid sequence.

Preferred examples of modified bTS02618A genes are shown in SEQ ID. Nos. 6 and 8, illustrating DNA sequences encoding the BTS02618A toxin and a variant thereof. These DNA sequences have an overall modified codon usage, which has been adapted to that of plants, particularly monocots such as corn. The DNA of SEQ ID. No. 6 encodes exactly the same toxin as the native bTS02618A gene, but yields higher expression levels in plants, particularly monocots such as corn, due to the adaptation of its codon usage to that of the plant host cells.

Furthermore, the BTS02618Aa toxin was found to bind to a receptor different from the CryIAb toxin receptor population in Ostrinia nubilalis gut membranes. This indicates that the BTS02618A toxin has a unique receptor in its susceptible insects. The broad spectrum, the binding to a different receptor and the low homology with other Bt toxins indicates that the BTS02618A toxin represents a new class of Bt toxins. Since the BTS02618A toxin apparently recognizes a different target site, it can prove to be especially useful for preventing the development of insect resistance, or for combatting insects resistant to other Bt toxins. Particularly the combined expression of the bTS02618A gene with other Bt genes encoding non-competitively binding toxins (as described in EP 408 403) in one host is interesting for preventing resistance development, preferably the combined expression of CryIAb and BTS02618A proteins.

Because of the broad spectrum of susceptible pest insects, the BTS02618A toxin and its variants are extremely useful for

transforming plants, e.g., monocots such as corn and vegetables such as Brassicas, to protect these plants from insect damage.

The insecticidally effective bTS02618A gene part or its equivalent, preferably the bTS02618A chimeric gene, encoding an insecticidally effective portion of the BTS02618A protoxin, can be stably inserted in a conventional manner into the nuclear genome of a single plant cell, and the so-transformed plant cell can be used in a conventional manner to produce a transformed plant that is insect-resistant. In this regard, a disarmed Ti-plasmid, containing the insecticidally effective bTS02618A gene part, in Agrobacterium tumefaciens can be used to transform the plant cell, and thereafter, a transformed plant can be regenerated from the transformed plant cell using the procedures described, for example, in EP 0,116,718, EP 0,270,822, PCT publication WO 84/02,913 and European Patent Application ("EPA") 87/400,544.0 (which are also incorporated herein by reference), and in Gould et al. (1991). Preferred Ti-plasmid vectors each contain the insecticidally effective bTS02618A gene part between the border sequences, or at least located to the left of the right border sequence, of the T-DNA of the Ti-plasmid. Of course, other types of vectors can be used to transform the plant cell, using procedures such as direct gene transfer (as described, for example in EP 0,233,247), pollen mediated transformation (as described, for example in EP 0,270,356, PCT publication WO 85/01856, and US Patent 4,684,611), plant RNA virus-mediated transformation (as described, for example in EP 0,067,553 and US Patent 4,407,956), liposome-mediated transformation (as described, for example in US Patent 4,536,475), and other methods such as the recently described methods for transforming certain lines of corn (Fromm et al., 1990; Gordon-Kamm et al., 1990) and rice (Shimamoto et al., 1989; Datta et al., 1990) and the recently described method for transforming monocots generally (PCT publication WO 92/09696).

The resulting transformed plant can be used in a conventional plant breeding scheme to produce more transformed plants with the same characteristics or to introduce the

insecticidally effective bTS02618A gene part in other varieties of the same or related plant species. Seeds, which are obtained from the transformed plants, contain the insecticidally effective bTS02618A gene part as a stable genomic insert. Cells of the transformed plant can be cultured in a conventional manner to produce the insecticidally effective portion of the BTS02618A protoxin, preferably the BTS02618A toxin, which can be recovered for use in conventional insecticide compositions against Lepidoptera (U.S. Patent Application 821,582; EPA 86/300291.1.).

The insecticidally effective bTS02618A gene part, preferably the truncated bTS02618A gene, is inserted in a plant cell genome so that the inserted gene is downstream (i.e., 3') of, and under the control of, a promoter which can direct the expression of the gene part in the plant cell. This is preferably accomplished by inserting the bTS02618A chimeric gene in the plant cell genome. Preferred promoters include: the strong constitutive 35S promoters (the "35S promoters") of the cauliflower mosaic virus of isolates CM 1841 (Gardner et al., 1981), CabbB-S (Franck et al., 1980) and CabbB-JI (Hull and Howell, 1987); and the TR1' promoter and the TR2' promoter (the "TR1' promoter" and "TR2' promoter", respectively) which drive the expression of the 1' and 2' genes, respectively, of the T-DNA (Velten et al., 1984). Alternatively, a promoter can be utilized which is not constitutive but rather is specific for one or more tissues or organs of the plant (e.g., leaves and/or roots) whereby the inserted bTS02618A gene part is expressed only in cells of the specific tissue(s) or organ(s). For example, the insecticidally effective bTS02618A gene part could be selectively expressed in the leaves of a plant (e.g., corn, cotton) by placing the insecticidally effective gene part under the control of a light-inducible promoter such as the promoter of the ribulose-1,5-bisphosphate carboxylase small subunit gene of the plant itself or of another plant such as pea as disclosed in U.S. Patent Application 821,582 and EPA 86/300,291.1. Another alternative is to use a promoter whose expression is inducible (e.g., by temperature or chemical

factors).

The insecticidally effective bTS02618A gene part is inserted in the plant genome so that the inserted gene part is upstream (i.e., 5') of suitable 3' end transcription regulation signals (i.e., transcript formation and polyadenylation signals). This is preferably accomplished by inserting the bTS02618A chimeric gene in the plant cell genome. Preferred polyadenylation and transcript formation signals include those of the octopine synthase gene (Gielen et al., 1984) and the T-DNA gene 7 (Velten and Schell, 1985), which act as 3'-untranslated DNA sequences in transformed plant cells.

The insecticidally effective bTS02618A gene part can optionally be inserted in the plant genome as a hybrid gene (EPA 86/300,291.1; Vaeck et al., 1987) under the control of the same promoter as a selectable marker gene, such as the neo gene (EP 0,242,236) encoding kanamycin resistance, so that the plant expresses a fusion protein.

All or part of the bTS02618A gene, encoding an anti-lepidopteran protein, can also be used to transform other bacteria, such as a B. thuringiensis which has insecticidal activity against Lepidoptera or Coleoptera. Thereby, a transformed Bt strain can be produced which is useful for combatting a wide spectrum of lepidopteran and coleopteran insect pests or for combatting additional lepidopteran insect pests. Transformation of bacteria with all or part of the bTS02618A gene, incorporated in a suitable cloning vehicle, can be carried out in a conventional manner, preferably using conventional electroporation techniques as described in Mahillon et al. (1989) and in PCT Patent publication WO 90/06999.

Alternatively, mutants of the BTS02618A, BTS02617A, BTS02654B and BTS02652E strains can be obtained by treating these strains with mutagenic agents such as nitrosoguanidine or with UV light; techniques which are well known to those skilled in the art. Also, asporogenous mutants can be obtained by treatment with ethylmethane sulfonate. Such mutants can be screened for improved characteristics (such as suitability for

large-scale fermentation and the like), while retaining substantially the same insecticidal activity.

The BTS02617A, BTS02618A, BTS02654B or BTS02652E strain also can be transformed with all or an insecticidally effective part of one or more foreign Bt genes such as: the bt18 gene (EP 0,358,557) or another Bt gene coding for an anti-Lepidoptera protein; and the bt109P gene (PCT publication WO 91/16433), coding for an anti-Coleoptera protein. Thereby, a transformed Bt strain can be produced which is useful for combatting an even greater variety of insect pests (e.g., Coleoptera and/or additional Lepidoptera).

Transformation of the BTS02617A, BTS02618A, BTS02654B or BTS02652E strain with all or part of a foreign Bt gene, incorporated in a conventional cloning vector, can be carried out in a well known manner, preferably using conventional electroporation techniques (Chassy et al., 1988) or other methods, e.g., as described by Lereclus et al. (1992).

Each of the BTS02617A, BTS02618A, BTS02654B or BTS02652E strains can be fermented by conventional methods (Dulmage, 1981; Bernhard and Utz, 1993) to provide high yields of cells. Under appropriate conditions which are well understood (Dulmage, 1981), the BTS02617A, BTS02618A, BTS02654B and BTS02652E strains each sporulate to produce crystal proteins containing the BTS02168A protoxin in high yields.

An insecticidal, particularly anti-lepidopteran, composition of this invention can be formulated in a conventional manner using the BTS02617A, BTS02618A, BTS02654B or BTS02652E strain or preferably their respective crystals, crystal proteins or the BTS02168A protoxin, toxin or insecticidally effective protoxin portion as an active ingredient, together with suitable carriers, diluents, emulsifiers and/or dispersants (e.g., as described by Bernhard and Utz, 1993). This insecticide composition can be formulated as a wettable powder, pellets, granules or dust or as a liquid formulation with aqueous or non-aqueous solvents as a foam, gel, suspension, concentrate, etc. The concentration of the BTS02617A, BTS02618A, BTS02654B or BTS02652E strain, crystals,

crystal proteins, or the BTS02618A protoxin, toxin or insecticidally effective protoxin portions in such a composition will depend upon the nature of the formulation and its intended mode of use. Generally, an insecticide composition of this invention can be used to protect a field for 2 to 4 weeks against Lepidoptera with each application of the composition. For more extended protection (e.g., for a whole growing season), additional amounts of the composition should be applied periodically.

A method for controlling insects, particularly Lepidoptera, in accordance with this invention preferably comprises applying (e.g., spraying), to a locus (area) to be protected, an insecticidal amount of the BTS02617A, BTS02618A, BTS02654B or BTS02652E strain, spores, crystals, crystal proteins or the BTS02168A protoxin, toxin or insecticidally effective protoxin portions, preferably the BTS2168A toxin. The locus to be protected can include, for example, the habitat of the insect pests or growing vegetation or an area where vegetation is to be grown.

To obtain the BTS02618A protoxin or toxin, cells of the BTS02617A, BTS02618A, BTS02654B or BTS02652E strain can be grown in a conventional manner on a suitable culture medium and then lysed using conventional means such as enzymatic degradation or detergents or the like. The protoxin can then be separated and purified by standard techniques such as chromatography, extraction, electrophoresis, or the like. The toxin can then be obtained by trypsin digestion of the protoxin.

The BTS02617A, BTS02618A, BTS02654B or BTS02652E cells can also be harvested and then applied intact, either alive or dead, preferably dried, to the locus to be protected. In this regard, it is preferred that a purified BTS02617A, BTS02618A, BTS02654B or BTS02652E strain (either alive or dead) be used, particularly a cell mass that is 90.0 to 99.9 % of the BTS02617A, BTS02618A, BTS02654B or BTS02652E strain.

The BTS02617A, BTS02618A, BTS02654B, or BTS02652E cells, crystals or crystal proteins or the BTS02618A protoxin, toxin,

or insecticidally effective protoxin portion can be formulated in an insecticidal composition in a variety of ways, using any number of conventional additives, wet or dry, depending upon the particular use. Additives can include wetting agents, detergents, stabilizers, adhering agents, spreading agents and extenders. Examples of such a composition include pastes, dusting powders, wettable powders, granules, baits and aerosol sprays. Other Bt cells, crystals, crystal proteins, protoxins, toxins, and insecticidally effective protoxin portions and other insecticides, as well as fungicides, biocides, herbicides and fertilizers, can be employed along with the BTS02617A, BTS02618A, BTS02654B or BTS02652E cells, crystals or crystal proteins or the BTS02618A protoxin, toxin or insecticidally effective protoxin portions to provide additional advantages or benefits. Such an insecticidal composition can be prepared in a conventional manner, and the amount of the BTS02617A, BTS02618A, BTS02654B or BTS02652E cells, crystals or crystal proteins or the BTS02618A protoxin, toxin or insecticidally effective protoxin portion employed depends upon a variety of factors, such as the insect pest targeted, the composition used, the type of area to which the composition is to be applied, and the prevailing weather conditions. Generally, the concentration of the BTS02618A protoxin, insecticidally effective protoxin portions or toxin will be at least about 0.1% by weight of the formulation to about 100% by weight of the formulation, more often from about 0.15% to about 0.8% by weight of the formulation.

In practice, some insects can be fed the BTS02618A protoxin, toxin, insecticidally effective protoxin portion or mixtures thereof in the protected area, that is in the area where such protoxin, toxin and/or insecticidally effective protoxin portion has been applied. Alternatively, some insects can be fed intact and alive cells of the BTS02617A, BTS02618A, BTS02654B or BTS02652E strain or transformants thereof, so that the insects ingest some of the strain's protoxin and suffer death or damage.

For th purpose of combatting insects by contacting them

with the BTS02618A protein, e.g., in the form of transformed plants or insecticidal formulations and the like, any of the above described variants of the BTS02618A protein with substantially the same insecticidal activity can be used, preferably the BTS02618Aa and BTS02618Ab proteins. Furthermore, any of the above-described methods for transforming plants and bacteria can also be utilized to combat insects with the BTS02618Aa or BTS02618Ab proteins or other more protease resistant protein variants of the BTS02618A protein in lieu of the native BTS02618A protein.

The following Examples illustrate the invention. The figure and the sequence listing referred to in the Examples are as follows:

Figure 1

Southern blot analysis of AluI-digested total DNA of Bt strain HD127 (lane 1), the BTS02618A strain (lane 2), Bt strain BTS02459 (containing cryIA(c), 81k, cryIC en cryIE, lane 3), and Bt strain BTS02480E (containing the same genes as HD-127, lane 4), using a mixture of DNA-probes for cryI crystal protein genes, including the cryIG probe (SEQ ID no. 1). Each band corresponds to a particular crystal protein gene. With these probes, the BTS02618A strain is found to contain the cryIA(b) gene and a novel gene, which is the bTS02618A gene, identified by an AluI fragment of approximately 530 bp, hybridizing to the cryIG probe of SEQ ID no. 1. The names of the recognized cryI genes are indicated, as well as the size of some fragments. The bTS02618A gene is indicated with three asterisks; "?" indicates an unknown gene fragment.

Sequence Listing

SEQ ID No. 1 - Nucleotide sequence of the DNA probe used to isolate the bTS02618A gene. This probe is derived from part of the cryIG DNA sequence and is complementary to nucleotides 2732-2750 of the DNA sequence described by Smulevitch et al. (1991).

SEQ ID No. 2 - The 5' partial nucleotide sequence of the bTS02618A gene, comprising the presumptive translation

initiation codon at nucleotide position 195-197.

SEQ ID No. 3 - The 3' partial nucleotide sequence of the bTS02618A gene (N: unknown nucleotide), comprising the presumptive translational stop codon at nucleotide position 1146-1148.

SEQ ID No. 4 - The nucleotide sequence of the bTS02618A gene and the translated amino acid sequence of the BTS02618A protoxin. The open reading frame of the protoxin reaches from nucleotide 668 to nucleotide 4141. The translation initiation codon is at nucleotide position 668-670, the translation stop codon is at nucleotide position 4139-4141.

SEQ ID. No. 5 - The amino acid sequence of the BTS02618A protein. The sequence of the about 69 kD BTS02618A toxin stretches from amino acid 44 to amino acid 658.

SEQ ID. No. 6 - The nucleotide sequence of a modified truncated bTS02618A gene, and the translated amino acid sequence of the BTS02618A toxin.

SEQ ID. No. 7 - The translated amino acid sequence of the modified bTS02618A toxin gene. Although only the toxin part is shown here, the full length protein is 100 % identical in amino acid sequence to the BTS02618A protein (SEQ ID. No.5).

SEQ ID. No. 8 - The nucleotide sequence of the modified bTS02618Aa toxin gene, and the translated amino acid sequence of the BTS02618Aa toxin. Besides N- and C-terminal amino acid deletions and the addition of an Alanine codon after the N-terminal Methionine codon, the BTS02618Aa toxin only differs from the BTS02618A toxin in amino acid number 123 (Arg codon has been changed into a Lys codon).

SEQ ID. No. 9 - The amino acid sequence of the BTS02618Aa toxin. The BTS02618Aa protoxin is 100 % identical to the BTS02618A protoxin in its part C-terminal from the toxin fragment.

Unless otherwise stated in the Examples, all procedures

for making and manipulating recombinant DNA are carried out by the standardized procedures described in Sambrook et al., Molecular Cloning - A Laboratory Manual, Second Ed., Cold Spring Harbor Laboratory Press, NY (1989).

Example 1: Characterization of the BTS02617A, BTS02618A, BTS02654B and BTS02652E strains.

The BTS02617A, the BTS02618A and the BTS02654B strain were isolated from grain dust sampled in Cadlan, province of Bicol, The Philippines and were deposited at the BCCM-LMG on July 2, 1992 under accession Nos. LMG P-12592, LMG P-12593 and LMG P-12594, respectively. Strain BTS02652E was also isolated from Philippine grain dust, and was deposited at the BCCM-LMG on March, 1, 1993 under accession No. LMG P-13493.

Each strain can be cultivated on conventional standard media, preferably T_3 medium (tryptone 3 g/l, tryptose 2 g/l, yeast extract 1.5 g/l, 5 mg $MnCl_2$, 0.05 M Na_2PO_4, pH 6.8 and 1.5% agar), preferably at 28°C. For long term storage, it is preferred to mix an equal volume of a spore-crystal suspension with an equal volume of 50% glycerol and store this at -70°C or lyophilize a spore-crystal suspension. For sporulation, growth on T_3 medium is preferred for 48 hours at 28°C, followed by storage at 4°C. During its vegetative phase, each of the strains can also grow under facultative anaerobic conditions, but sporulation only occurs under aerobic conditions.

Sterilization of each strain occurs by autoclave treatment at 120°C (1 bar pressure) for 20 minutes. Such treatment totally inactivates the spores and the BTS02617A,BTS02618A, BTS02654B, and BTS02652E protoxins. UV radiation (254 nm) also inactivates the spores.

After cultivating on Nutrient Agar ("NA", Difco Laboratories, Detroit, MI, USA) for one day, colonies of each of the BTS02617A, BTS02618A, BTS02654B and BTS02652E strains form opaque white colonies with irregular edges. Cells of each strain (Gram positive rods of 1.7-2.4 x 5.6-7.7 μm) sporulate after 48 hrs cultivation at 28°C on T_3 agar. The crystal

proteins produced during sporulation are packaged in crystals of the BTS02617A, BTS02618A, BTS02654B, and BTS02652E strains. Quite remarkably, the crystal remains attached to the spore after sporulation.

The Bt serotype of the BTS02617A, BTS02618A, BTS02654B and BTS02652E strains was determined to be serotype tolworthi H9 of all these strains which was determined by conventional serotyping methods as conducted by the WHO Collaborating Center for Entomopathogenic Bacillus.

Example 2 : Insecticidal activity of the BTS02617A, BTS02618A, BTS02654B and BTS02652E strains, the BTS02618A protoxin and the BTS02618Aa and BTS02618Ab toxins or protoxins against Noctuidae spp., Gelechiidae spp., Yponomeutidae spp. and Pyralidae spp.

Toxicity assays were performed on neonate larvae (for Plutella xylostella, third instar larvae were used) fed on an artificial diet layered with spore-crystal mixtures from one of the BTS02617A, BTS02618A, BTS02654B and BTS02652E strains or the BTS02618A protoxin or toxin or the BTS02618Aa and BTS02618Ab toxins or protoxins. The artificial diet was dispensed in wells of Costar 24-well plates. Formaldehyde was omitted from the diet. 50 µl of a sample dilution was applied on the surface of the diet and dried in a laminar air flow. For LC_{50} assays, the dilutions were made in a PBS-BSA buffer, and five dilutions were applied. Two larvae were placed in each well and 24 larvae were used per sample dilution. Dead and living M. brassica, S. frugiperda, H. virescens, O. nubilalis, Plutella xylostella and S. exigua larvae were counted on the fifth day, and dead and living A. ipsilon, A. segetum and S. littoralis larvae were counted on the sixth day. The LC_{50} and LC_{95} values (the concentrations required to kill respectively 50% or 95% of the insects tested, expressed in number of spore-crystals/cm^2 or ng (pro)toxin/cm^2) were calculated using Probit-analysis (Finney, 1971), and the results are set forth below.

The potato moth, Phthorimaea operculella, was tested by the following assay: disks, cut from potato tubers, were dipped

in solutions of varying concentrations of BTS0216Aa protein. Three of such disks, which were allowed to dry, were placed in a tray with 20 Phthorimaea larvae. Mortality was recorded after 4 to 5 days for each concentration applied.

Spodoptera littoralis

Experiment/Strain	LC_{50} [a]	LC_{95} [a]	$FL_{min-max}$ [b]	Slope
Experiment 1				
BTS02618A	2.4	7.7	1.5-3.4	3.2
HD127[c]	2.5	168	1.2-7.4	1.0
Experiment 2				
BTS02618A	1.1	4	0.8-1.6	3.0
HD127	21.2	133.7	14.4-31.9	2.0

[a] 10^5 spore-crystals per cm^2

[b] 95 % fiducial limits of LC_{50} values

[c] from the Howard Dulmage collection, housed at the Northern Region Research Center, 1815 North University, Peoria, Ill, USA. The curator is Dr. L.Nakamura.

Experiments with purified BTS02618A protoxin also show a significant toxicity of this protoxin against S. littoralis larvae.

Spodoptera exigua

1. Crystal/spore mixtures

Experiment/Strain	LC_{50} [a]	LC_{95} [a]	$FL_{min-max}$ [b]	Slope
Experiment 1				
BTS02618A	1.4	7.9	0.48-3.9	2.2
HD127	8.2	163.5	5.1-15.7	1.3
Experiment 2				
BTS02618A	1.2	3.56	0.91-1.57	3.5
BTS02617A	0.79	2.12	0.61-1.03	3.81
HD127	3.5	44.2	1.36-11.5*	1.5
Florbac	4.1	53.9	1.5-17.0*	1.47
BTS00170U[c]	5.1	46.5	1.83-24.4*	1.71
Experiment 3				
Javelin[d]	23.12	195.7	14.6-56.7	1.77
Experiment 4				
BTS02618A	1.07	2.91	0.83-1.39	3.8
BTS02617A	0.87	4.7	0.59-1.21	2.22
HD127	4.7	56.9	1.85-18.7*	1.52
Florbac[e]	2.53	48.1	0.79-6.71*	1.29
BTS00170U	1.94	56.3	0.55-5.4*	1.12

[a] 10^5 spore-crystals per cm^2

[b] 95 % fiducial limits of LC_{50} values, values marked with * are 90 % fiducial limits of LC_{50} values

[c] PCT patent publication WO 90/06999

[d] strain isolated from Javelin® (Sandoz, Lichtstrasse, Basel, Switzerland)

[e] strain from Florbac (Novo Nordisk, Novo Allè, Bagsværd, Denmark)

2.Toxin/protoxin assays.

ICP		LC_{50} [a]	LC_{95} [a]	$FL_{min-max}$ [b]	Slope
BTS02618A	Protoxin	26.6	100.6	20.9-33.9	2.8
CryIC	Toxin	68.9	313.2	50.5-94.1	2.5
CryID	Toxin	118.6	870.6	82.7-170.0	1.9

[a] ng/cm^2

[b] 95 % fiducial limits of LC_{50} values

Mamestra brassica

1. Crystal/spore mixtures.

Experiment/Strain	LC_{50} [a]	LC_{95} [a]	$FL_{min-max}$ [b]	Slope
HD127	37.8	297.6	17.8-91.1	1.8
BTS02618A	8.6	59.6	6.0-12.2	1.9
BTS02617A	5.2	25.8	3.7-7.1	2.4
BTS02652E	12.9	44.2	9.7-17.2	3.0
BTS02654B	14.2	60.5	10.8-19.9	2.6

[a] 10^5 spore-crystals per cm^2

[b] 95 % fiducial limits of LC_{50} values

2. Protoxin assays.

ICP		LC_{50} [a]	LC_{95} [a]	$FL_{min-max}$ [b]	Slope
BTS02618A	Protoxin	25.3	125.1	19.3-33.2	2.4
CryIC	Protoxin	22.0	62.9	16.3-29.6	3.6
CryIA(b)	Protoxin	162.4	7169	93.2-283.1	1.0

[a] ng/cm^2

[b] 95 % fiducial limits of LC_{50} values

Agrotis ipsilon

1.Crystal/spore mixtures.

Strain	mortality[a]	genes[b]
Btgall.[c]	1/20	cryIF, cryIG, cryII, 81k
HD127[d]	2/20	cryIAa, cryIAb, cryIC, cryID, cryII, 81k
BTS02618A	16/20[e]	cryIAb, cryII, bTS02618A
Buffer	1/20	none

[a] number of 1st instar larvae killed after 6 days (10^7 spore-crystals per cm^2)

[b] genes known to be present in these strains

[c] Btgall. as described by Smulevitch et al (1991)

[d] HD127 is available at the Howard Dulmage Collection (NRRC, see above)

[e] surviving larvae show severe growth-inhibition

STRAIN	LC_{50}[a]	LC_{95}[a]	$FL_{min-max}$[b]	Slope
BTS02618A	84.4	207.9	65.9-109.6	4.2
HD127	>250			
BTS02617A	53.4	261.0	27.7-112.3	2.4

[a] 10^6 spores/cm^2

[b] 95 % fiducial limits of LC_{50} values

2. Toxin/protoxin assay.

ICP		LC_{50}[a]	LC_{95}[a]	$FL_{min-max}$[b]	Slope
CryIAc	Toxin	>1350			
BTS02618A	Protoxin	212.2	1973	168.1-267.9	1.7

[a] ng/cm^2

[b] 95 % fiducial limits of LC_{50} values

Since MacIntosh et al. (1990) described some activity of the CryIAc toxin towards A. ipsilon, purified CryIAc toxin was tested on this insect for comparison but did not cause any significant mortality of A. ipsilon.

Heliothis virescens

1.Crystal/spore mixture.

Experiment/Strain	LC_{50}[a]	LC_{95}[a]	$FL_{min-max}$[b]	Slope
BTS02617A	1.69	14.99	0.67-2.89	1.73
BTS02618A	2.71	25.4	0.88-6.99	1.69
BTS00170U[c]	15.1	398.7	8.3-41.2	1.15
Dipel[d]	2.99	14.11	1.25-7.76	2.45

[a] 10^3 spore-crystals per cm^2

[b] 95% fiducial limits of LC_{50} values

[c] PCT patent publication WO 90/06999

[d] strain isolated from Dipel™ (Abbott Laboratories, North Chicago, Ill., USA)

2.Toxin/protoxin assay.

ICP		LC_{50}[a]	$FL_{min-max}$[b]	LC_{95}[a]	Slope
BTS02618A	Protoxin	31.6	20-50	182.7	2.1
CryIAb	Toxin	7.2	4.9-10.5	169.1	1.2

[a] ng/cm2

[b] 95 % fiducial limits of LC_{50} values

Ostrinia nubilalis

1.Crystal/spore mixtures.

Experiment/Strain	LC_{50}[a]	LC_{95}[a]	$FL_{min-max}$[b]	Slope
BTS02617A	4.92	12.49	2.45-6.81	4.0
BTS02618A	6.17	39.7	2.93-9.74	2.0
Dipel[c]	>30			

[a] 10^5 spore-crystals per cm^2

[b] 95% fiducial limits of LC_{50} values

[c] strain isolated from Dipel™ (Abbott Laboratories)

2.Purified protoxin assay

ICP		100 % Mortality[a]
CryIAb	Toxin	1350
CryIB	Toxin	1350
BTS02618A	Protoxin	100

[a] concentration at which 100 % mortality was observed (in ng/cm^2)

The purified BTS02618A protoxin also showed a significant toxicity to Ostrinia nubilalis larvae, as compared with the CryI toxins that are most active against Ostrinia.

Plutella xylostella

Plutella xylostella larvae also showed significant mortality after application of purified BTS02618A toxin to their artificial diet in several experiments.

Spodoptera frugiperda

Crystal/spore mixtures of a bTS02618A gene-transformed crystal-minus Bt strain (Mahillon et al., 1989) were also found to significantly inhibit larval growth of S. frugiperda larvae in insect feeding trials.

Agrotis segetum

The cutworm Agrotis segetum was also found to be susceptible to the BTS02618Aa toxin. This variant of the BTS02618A protein killed 50 % of the Agrotis larvae at a concentration of BTS02618Aa toxin of 980 ng/cm^2 (=LC_{50}). In comparative assays, all other CryI toxins tested (CryIAb, CryIAc, CryIAa, CryIB, CryIC, CryID, CryIE (Hofte & Whiteley 1989; EP 358 557)) were found to have an LC_{50} of more than 15.000 ng/cm^2 for this insect. Agrotis segetum is an important pest insect on various crops.

Phthorimaea operculella

Also the potato tubermoth, Phthorimaea operculella, was found to be susceptible to the BTS02618Aa toxin. Larvae which ingested the BTS02618A toxin showed a significantly higher mortality rate than control larvae.

Furthermore, the BTS02618Aa toxin was tested on several insects and was found to have substantially the same insecticidal activity as the BTS02618A protein. Indeed, bio-assays were conducted with Heliothis virescens, Mamestra brassicae, Ostrinia nubilalis, Spodoptera exigua and Spodoptera littoralis, and these showed only minor differences in LC_{50} values when compared to the BTS02618A protein. This shows that the new BTS02618Aa toxin does not differ substantially in insecticidal activity from the native form.

Also, an Alanine mutant of the BTS02618A toxin and protoxin, the BTS02618Ab toxin and the BTS02618Ab protoxin, were tested on Ostrinia nubilalis and were found to be substantially as toxic as the BTS02618Aa toxin or protoxin.

At the same time, the BTS02618Aa toxin was found to be non-toxic to the tested Coleopteran insects: Leptinotarsa

decemlineata and Diabrotica undecimpunctata howardi were not affected by the BTS02618Aa toxin. These insects were tested in diet application assays well known in the art. See, for example, Rupar et al., 1991.

In conclusion, the strains of this invention and the BTS02618A protein of this invention and its variants have a strong insecticidal activity against a broad range of insects that are not susceptible to any single currently available Bt protein and have an activity against at least three Spodoptera spp. and against other Noctuidae, such as A. ipsilon, A. segetum, M. brassica and H. virescens, as well as against Pyralidae, such as O. nubilalis, Gelechiidae such as P. operculella and Yponomeutidae such as Plutella xylostella. These results are summarized and compared with results for other CryI genes (Van Frankenhuyzen, 1993) in Table 1 which shows the unique range of insects susceptible to the BTS02618A protein.

The same spectrum applies for the BTS02618Aa and BTS02618Ab toxins. So these new toxins can also be used for combatting insects, and they have the added advantage that they are more stable, due to their lower susceptibility to protease activity, since almost no about 55kD protein is formed.

Example 3 : Identification of the bTS02618A gene

The bTS02618A gene was identified in the BTS02618A strain by Southern blot analysis (Fig. 1) of AluI digested total DNA of the strain using, as a DNA probe, the DNA sequence of the cryIG gene (Gleave et al., 1992) of SEQ ID No. 1 and using standard hybridization conditions. Partial DNA sequences of the bTS02618A gene, showing its 5' and 3' end portions, are shown in SEQ ID Nos. 2 and 3, respectively, and the full DNA sequence of the bTS02618A gene and the full amino acid sequence of the BTS02618A protein are shown in SEQ ID No. 4.

The partial sequences of SEQ ID Nos. 2 and 3 allow the bTS02618A gene to be recognized in the BTS02617A, BTS02654B and BTS02652E strains and allow the construction of probes to identify and isolate the full gene sequence in these and oth r

Bt strains. The translation initiation codon of the bTS02618A gene is identified at nucleotide position 195-197 in SEQ ID No. 2, corresponding to nucleotide position 668-670 in SEQ ID No.4. The translation stop codon is identified at nucleotide position 1146-1148 in SEQ ID No. 3, corresponding to nucleotide position 4139-4141 in SEQ ID No. 4.

The bTS02618A gene was also identified in the BTS02617A, BTS02654B and BTS02652E strains by using the DNA sequence of SEQ ID No. 1 as a probe, as well as other DNA probes of conserved DNA fragments in cryI genes.

The full length bTS02618A gene was found to encode a 129.9 kD protoxin. A comparison of the amino acid sequence with other known CryI proteins showed that the C-terminal part (C-terminal of conserved sequence block 5) was homologous with CryIG (88%). The best homology for the N-terminal part (the toxin) was found with the CryIB toxin, but this was found to be less than 50% (homology is expressed as the number of perfect matches divided by the number of amino acids of the longest fragment).

The smallest insecticidal protein is believed to be a 69 kD (615 amino acids) protein stretching from amino acid number 44 to amino acid number 658 in SEQ ID No. 4. A smaller tryptic fragment of 55 kD (494 amino acids), stretching from amino acid number 165 to amino acid number 658 in SEQ ID No. 4, still has insecticidal activity towards S. exigua, but this activity is significantly reduced. Thus, a truncated bTS02618A gene or an equivalent truncated gene preferably encodes the 69 kD protein of the BTS02618A protoxin of SEQ ID No.4 as described above.

Example 4 : Cloning and expression of the bTS02618A gene

In order to isolate the bTS02618A gene, total DNA from the BTS02618A strain was prepared and partially digested with Sau3A. The digested DNA was size fractionated on a sucrose gradient and fragments ranging from 7 Kb to 10 Kb were ligated to the BamH1-digested and BAP-treated cloning vector pUC19 (Yannisch-Perron et al., 1985). Recombinant E.coli clones containing the vector were then screened with the cryIG DNA

probe of SEQ ID No. 1 which is described in Example 3, to identify clones containing the bTS02618A gene.

The so-identified DNA fragments were then sequenced according to Maxam and Gilbert (1980). Partial sequences of the bTS02618A gene are shown in SEQ ID Nos. 2 and 3, and a full sequence of the bTS02618A gene and the BTS02618A protein is shown in SEQ ID No. 4. Based on the DNA sequence analysis, the gene is cut with appropriate restriction enzymes to give the truncated bTS02618A gene encoding the BTS02618A toxin. Expression of the gene in E.coli was induced using standard procedures (Sambrook et al., 1989, supra).

The bTS02618A gene was also introduced by routine procedures into a crystal-minus Bt 1715 berliner strain and a Bt HD-1 kurstaki strain (the production strain of Dipel™ (Abbott Laboratories)) under the control of its own bacterial promoter, using an appropriate shuttle vector (Mahillon et al., 1988).

Spore-crystal mixtures of 2 transformants of Bt strain kurstaki HD-1 (containing the bTS02618A gene), the parental Bt kustaki HD-1 strain, the wild-type BTS02618A strain, the Bt 1715 berliner crystal-minus strain and one transformant of Bt 1715 berliner crystal-minus (containing the bTS02618A gene) were bioassayed on beet armyworm (Spodoptera exigua). Bioassays were performed as described in Example 2. The transformed Bt 1715 berliner crystal-minus (containing bTS02618A) was highly toxic to S. exigua (100% mortality at 4 x 10^4 spore-crystals per square cm of diet agar) while the Bt 1715 berliner crystal-minus was not toxic. The Bt kurstaki HD-1 (bTS02618A) transformants were (on average) 22 times (LC_{50}-level) to 76 times (LC_{95}-level) as toxic as the parental HD-1 (Table 2).

Similarly, the BTS02618Aa protein or other variants of the BTS02618A protein can be transferred to and expressed in a Bt strain by any method available in the art (Baum et al., 1991; Gamel & Piot, 1992; Lecadet et al., 1992), provided the vector used is compatible with the Bt host strain, and is stably maintained in the bacterial host. It is known that plasmid

vectors having replicon homology with the host strain are not suitable vectors (Gamel & Piot, 1992).

Example 5: Insertion of the bTS02618A gene and the truncated bTS02618A gene in E. coli and insertion of the truncated bTS02618A gene in plants.

In order to express the bTS02618A gene and the truncated bTS02618A gene of Example 4 in E. coli and in plants, different gene cassettes are made in E. coli according to the procedure described in EPA 86/300291.1 and EPA 88/402115.5.

To allow significant expression in plants, cassettes containing a) the truncated gene or b) a hybrid gene that is a fusion of i) the truncated gene and ii) the neo gene are each: inserted between the T-DNA border sequences of intermediate plant expression vectors as described in EPA 86/300291.1; fused to transcript formation and polyadenylation signals in the plant expression vectors; placed under the control of the constitutive promoter from cauliflower mosaic virus driving the 35S3 transcript (Hull and Howell, 1987) or the 2' promoter from the TR-DNA of the octopine Ti-plasmid (Velten et al., 1984); and fused to 3' end transcript formation and polyadenylation signals of the octopine synthase gene (Gielen et al., 1984).

Using standard procedures (Deblaere et al., 1985), the intermediate plant expression vectors, containing the truncated bTS02618A gene, are transferred into the Agrobacterium strain C58C1Rif^R (US Patent Application 821,582; EPA 86/300,291.1) carrying the disarmed Ti-plasmid pGV2260 (Vaeck et al., 1987). Selection for spectinomycin resistance yields cointegrated plasmids, consisting of pGV2260 and the respective intermediate plant expression vectors. Each of these recombinant Agrobacterium strains is then used to transform different cotton plants so that the truncated bTS02618A gene is contained in, and expressed by, different plant cells.

Example 6: Expression of the truncated bTS02618A gene in plants.

The insecticidal activity against Lepidoptera of the expression products of the truncated bTS02618A gene in leaves of transformed plants, generated from the transformed plant cells of Example 5, is evaluated by recording the growth rate and mortality of Agrotis and Spodoptera spp. larvae fed on these leaves. These results are compared with the growth rate of larvae fed leaves from untransformed plants. Toxicity assays against Agrotis and Spodoptera spp. are performed as described in EP 0,358,557, U.S. Patent Application 821,582 and EPA 86/300,291.1.

A significantly higher mortality rate is obtained among larvae fed on leaves of transformed plants containing the truncated bTS02618A gene and the truncated bTS02618A-neo hybrid gene than among larvae fed the leaves of untransformed plants. The transformed plants are also found to resist Ostrinia nubilalis, Mamestra brassica, Heliothis virescens and Plutella xylostella attack by their expression of the BTS02618A protein.

Needless to say, this invention is not limited to the BTS02617A strain (BCCM-LMG P-12592), the BTS02618A strain (BCCM-LMG P-12593), the BTS02654B strain (BCCM-LMG P-12594) and the BTS02652E (BCCM-LMG P-13493) strain. Rather, the invention also includes any mutant or variant of the BTS02617A, BTS02618A, BTS02654B, and BTS02652E strain which produces crystals, crystal proteins, protoxin or toxin having substantially the same properties, particularly anti-Lepidoptera properties, quite particularly anti-Noctuidae, anti-Yponomeutidae, anti-Gelechiidae and anti-Pyralidae properties, especially anti-Spodoptera, anti-Plutella, anti-Ostrinia, anti-Mamestra; anti-Heliothis, anti-Phthorimaea and anti-Agrotis properties, as the respective BTS02617A, BTS02618A, BTS02654B or BTS02652E crystals or crystal proteins, or the BTS02618A protoxin or toxin. This invention also includes the bTS02618A gene and any insecticidally effective parts thereof, like the truncated bTS02618A gene. In this regard, the term "bTS02618A gene" as used herein means the gene isolated from the BTS02617A, BTS02618A, BTS02654B or BTS02652E strain and hybridizing to the nucleotide sequence of SEQ ID No.

1 and any equivalent gene encoding a protoxin having substantially the same amino acid sequence and insecticidal activity as the BTS02618A protoxin and preferably containing the partial nucleotide sequences shown in SEQ ID Nos. 2 and 3, or the full sequence shown in SEQ ID No. 4.

This invention also is not limited to cotton plants transformed with the truncated bTS02618A gene. It includes any plant, such as tomato, tobacco, rapeseed, alfalfa, sunflower, lettuce, potato, corn, rice, soybean, Brassica species, sugar beet and other legumes and vegetables, transformed with an insecticidally effective part of the bTS02618A gene or an equivalent gene such as the bTS02618Aa gene.

Methods for transforming corn cells and regenerating transgenic plants have been described (D'Halluin et al., 1992; Fromm et al., 1990; Gould et al., 1991; Koziel et al., 1993; Omirulleh et al.,1993; Spencer et al., 1992; Klein et al., 1992; Walters et al., 1992). Vectors for transforming corn cells contain chimeric genes encoding a bTS02618A protein or variant thereof (e.g., BTS02618Aa), comprising suitable promoters such as derivatives of the 35S promoter or a promoter from a maize gene, preferably a constitutively expressed maize gene; and suitable 3' end formation sequences such as those of natural maize genes or those derived from the 35S, gene7 or octopine synthase genes (See, Detailed Description and Mogen et al 1990; Wu et al 1993). Increased expression may be obtained when an intron is introduced in the chimeric gene construct (e.g. a natural maize gene intron such as the AdhI intron (see Callis et al., 1987; Maas et al., 1991)), provided the intron is correctly spliced in the corn cells. Enhancer elements can also be provided in the promoter sequence (e.g., Omirulleh et al., 1993). Transgenic corn plants are grown on selective media by inclusion of well known selectable marker genes such as herbicide resistance genes or antibiotic resistance genes as chimeric genes in the transforming DNA. Transgenic corn plants are selected for their transformed phenotype by means of bio-assays on Ostrinia nubilalis larvae. These larvae quickly stop feeding on the bTS02618Aa-transformed

corn and cause no major damage to the corn plants.

This invention is not limited to the use of Agrobacterium tumefaciens Ti-plasmids for transforming plant cells with an insecticidally effective bTS02618A gene part. Other known techniques for plant cell transformations, such as by means of liposomes, by electroporation or by vector systems based on plant viruses or pollen, can be used for transforming monocotyledons and dicotyledons with such a gene part.

Furthermore, DNA sequences other than those present naturally in the BTS02617A, BTS02618A, BTS02654B and BTS02652E strains and encoding the BTS02618A protoxin and toxin can be used for transforming plants and bacteria. In this regard, the natural DNA sequence of these genes can be modified by: 1) replacing some codons with others that code either for the same or different, preferably the same, amino acids; 2) deleting or adding some codons; and/or 3) reciprocal recombination as described by Ge et al. (1991); provided that such modifications do not substantially alter the properties, particularly the insecticidal properties, especially anti-lepidoptera properties, of the encoded, insecticidally effective portions of the BTS02618A protoxin (e.g., toxin). For example, an artificial bTS02618A gene or gene part of this invention, as described above, having a modified codon usage, could be used in certain circumstances instead of a natural insecticidally effective bTS02618A gene part in a bTS02618A chimeric gene of this invention for transforming plants.

Also, other DNA recombinants containing all or part of the bTS02618A gene in association with other foreign DNA, particularly the DNA of vectors suitable for transforming plants and microorganisms other than E. coli, are encompassed by this invention. In this regard, this invention is not limited to the specific plasmids containing the bTS02618A gene, or parts thereof, that were heretofore described, but rather, this invention encompasses any DNA recombinants containing DNA sequences that are their equivalent. Further, the invention relates to all DNA recombinants that include all or part of the bTS02618A gene and that are suitable for transforming

microorganisms (e.g., plant associated bacteria such as other Bacillus thuringiensis strains, Bacillus subtilis, Pseudomonas, and Xanthomonas or yeasts such as Streptomyces cerevisiae) under conditions which enable all or part of the gene to be expressed and to be recoverable from said microorganisms or to be transferred to a plant cell.

Example 7. Construction of an artificial bTS02618A gene, encoding the BTS02618A toxin.

Based on the amino acid sequence of the BTS02618A toxin, an artificial DNA sequence encoding substantially the same protein was designed. At first, a DNA sequence for the artificial BTS02618A toxin gene was designed, using corn-preferred codons (Murray et al., 1989). During the design of the artificial gene, TA and CG doublets at codon position 2 and 3 were avoided. The artificial gene was also corrected for local high GC-content (GC stretches of more than 5 bp were avoided). Also, suitable restriction sites were incorporated throughout the gene. So the final gene did not always use the most preferred corn codons. The artificial gene was synthesized on an Applied Biosystems 380B DNA synthesizer using standard cyanoethyl phoshoramidite chemistry. The oligonucleotides were gel purified and assembled into full length fragments using known techniques. See also, the method of Davies et al. (1991). The artificial toxin gene also carried a deletion of codon 2 to codon 43 of the BTS02618A coding sequence, and codon 44 is preceded by an ATG (start) and GCT (Ala) codon, to create a suitable translation initiation context as proposed by Joshi (1987). The C-terminal end of the artificial bTS02618A toxin gene contained some codons in addition to the determined minimal toxic gene fragment, because of the presence of a suitable maize translational stop context in this further C-terminal part.

The chimeric gene construct containing the artificial bTS02618A gene is introduced into corn cells as described above. Most of the corn plants that are regenerated from these

cells and that are identified as transformed are insecticidal because of expression of the bTS02618A gene. Northern and Southern analysis of some selected transgenic corn plants show the stable integration of the transgene and the presence of readily detectable levels of BTS02618A mRNA expression. These plants also show good insect control, and the degree of insecticidal activity is linked to the quantity of the Bt protein present in the tissues, as determined by ELISA.

It is believed that any method known for transforming corn so that it expresses the BTS02618A protein at sufficient levels can be used to develop insect-resistant corn. In this regard, it may preferred to express at least 2 non-competitively binding Bt proteins, such as CryIAb and BTS02618A in one plant to prevent the development of insect resistance.

Example 8. Design of a new variant of the BTS02618A protein

To prevent further proteolytic cleavage of the BTS02618A about 69 kD toxin, a new variant of the BTS02618A protein was made. In one new variant of this protein, the Arg at amino acid position 123 in SEQ ID. No. 6 was replaced with a Lys (the BTS02618Aa protein). In another variant, the Arg at position 123 in SEQ ID. No. 6 was replaced by Ala (the BTS02618Ab protein). These proteins were found to be more resistant to protease treatment (i.e., the proteins yielded no about 55kD protein) and insect assays confirmed that their toxicity was retained. The amino acid sequence of the BTS02618Aa toxin is shown in SEQ ID. No. 9.

Other examples having the amino acid sequence changes around the protease cleavage site are made and are also found to have more resistance to protease activity, while retaining their insecticidal activity.

Also, an artificial gene encoding a BTS02618Aa toxin fragment was designed. Besides N- and C-terminal deletions and the addition of a Met and an Ala codon at positions 1 and 2 (as for the DNA of SEQ ID. No. 6), this gene differs in one codon from the synthetic gene of SEQ ID. No. 6: the Arg codon (CGC)

was replaced by a Lys (AAG) codon. The other nucleotides were the same as for the artificial bTS02618A gene of SEQ ID. No. 6. Such a modification was made by PCR-mediated mutagenesis, starting from the bTS02618A artificial gene, using the appropriate primers. Essentially, a PCR-generated and restriction enzyme-digested fragment having the mutated codon at position 123 was inserted into the corresponding site of the digested bTS02618A gene of SEQ ID. No. 6 to give the DNA of SEQ ID. No. 8.

Corn plants are also transformed with the bTS02618Aa gene, following the procedures described above. Selected transformed corn plants expressing the bTS02618Aa gene are insecticidal for Ostrinia nubilalis larvae. It is believed that any method described in the Detailed Description can be used for expressing the above Bt genes in transformed corn plants, either alone or in combination with other Bt genes. A particularly preferred candidate is a DNA sequence encoding the CryIAb protein. Following routine procedures, appropriate lines having desired qualities can be selected between the obtained regenerants.

Example 9. Binding of BTS02618A toxin to insect gut membranes.

The BTS02618Aa toxin was found not to inhibit binding of the CryIAb toxin to midgut membrane vesicles of Ostrinia nubilalis.

In this experimental setup, the proteins used were: the Lysine mutant of BTS02618A (BTS02618Aa), non-biotinylated; CryIAb, non-biotinylated; and biotinylated (and biologically active) CryIAb . All ICPs used were trypsin resistant toxins. The following combinations were tested:

Biotinylated CryIAb x no competitor;

Biotinylated CryIAb x 1000-fold excess of CryIAb toxin;

Biotinylated CryIAb x 1000-fold excess of BTS02618Aa toxin.

For these experiments, 10 ng biotinylated CryIAb, with or without an excess of an unlabeled crystal protein, was mixed with 10 microgram brush border membrane vesicles derived from

larval midguts of Ostrinia nubilalis. These vesicles were prepared according to the method of Wolfersberger et al. (1987). These mixtures were made in PBS (8 mM Na2HPO4, 2 mM KH2PO4, 150 mM NaCl, pH 7.4) containing 0.1% BSA. The mixtures were incubated during 1 hour at room temperature and were then centrifuged for 10 minutes. After washing the pellet in 500 microliter PBS-0.1% BSA, the pellet was centrifuged again and dissolved in sample buffer for SDS-PAGE. The samples were run on a 10 % polyacrylamide gel. The gel was blotted at room temperature during two hours on a semi-dry blotting apparatus (LKB Novablot; the blotting buffer used was: 39 mM glycine, 48 mM Tris, 0.0375 % (w/v) sodium dodecyl sulphate, 20 % methanol). The membrane was blocked for at least 2 hours in TBS (10 mM Tris, 150 mM NaCl, pH 7,6) with 0.1% BSA, followed by incubation with a streptavidin-peroxidase conjugate, diluted 1/1000 in TBS-0.1% BSA for 45 minutes. The membrane was washed for 4 times 5 minutes and once for 15 minutes with TBS- 0.2% Tween 20. Between the wash steps, the blot was thoroughly washed under the tap. The membrane was incubated in ECL reagent (Amersham) for 1 minute and was then exposed to X-ray film.

For the biotinylated CryIAb, a band corresponding to bound toxin was observed on the X-ray film. When biotinylated CryIAb toxin was incubated in the presence of excess CryIAb toxin, no band was observed on the film: as expected, the excess unlabeled toxin had displaced the labeled toxin. For the biotinylated CryIAb toxin in the presence of an excess of the BTS02618Aa toxin, a band corresponding to bound biotinylated CryIAb was seen: unlabeled BTS02618Aa toxin was apparently unable to compete with CryIAb for binding to the vesicles, indicating that BTS02618Aa binds to another receptor other than CryIAb in Ostrinia nubilalis.

In a similar setup, unlabeled CryIAb toxin did not compete for the receptors of biotin-labeled and biologically active BTS02618Aa toxin, while such competition was observed with an excess of unlabeled BTS02618Aa toxin.

Thus, the BTS02618A protein recognizes a different receptor

site in Ostrinia midgut membranes, and can be used in a strategy to delay or prevent the development of insect resistance or to combat insects resistant to the CryIAb toxin, e.g., by expressing the CryIAb and the BTS02618A toxin in a plant. Since both toxins are highly active against a group of major insect pests and apparently recognize different receptor molecules, their use in transgenic plants such as corn and vegetables, provides a supplemental advantage. Corn plants can be transformed with the cryIAb and bTS02618Aa gene with any method available in the art, such as crossing plants expressing either toxin, or any of the methods described in EP publication number 408 403.

Table 1.

Activity of CryI proteins towards several lepidopteran insect pests: + and - indicates the presence or absence of insecticidal activity, +/- indicates low activity (according to Van Frankenhuyzen (1993)), NA indicates no data available, the protein BTS02618A is abbreviated as 2618A (data of Van Frankenhuyzen (1993) and this invention (for A. ipsilon and 2618A)).

	2618A	IAb	IAc	IB	IC	IF
S.exigua	+	+/-	-	-	+	+
S.littoralis	+	-	-	-	+	NA
H.virescens	+	+	+	-	+/-	+
A.ipsilon	+	NA	-	NA	NA	NA
O.nubilalis	+	+	+	NA	NA	+
P.xylostella	+	+	+	+	+	NA
M.brassica	+	+	-	-	+	NA

Table 2.

LC_{50}-LC_{95} assays with spore-crystal mixtures of recombinant Bt's. Tests were performed as described in the text. Values

indicate the number of spore-crystals x 10^6 per square cm of diet agar.

Strain	LC_{50}	LC_{95}	F195min-max	Slope
Bt kurstaki HD-1	8.9	91.2	3.9-15.4	1.6 ± 0.4
HD-1/1 (bTS02618A)	0.4	1.7	0.1 - 0.6	2.5 ± 0.7
HD-1/2 (bTS02618A)	0.4	0.93	0.2 - 0.5	4.3 ± 1.2
BTS02618A	1.5	4.3	0.9 - 2.1	3.6 ± 0.9

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Claims:

1. A variant of a BTS02618A protein comprising a BTS02618A protoxin or an insecticidally effective portion thereof, wherein said protoxin or said insecticidally effective portion thereof is modified in the amino acid sequence thereby making said variant more resistant to protease whereby said variant retains substantial insecticidal activity upon protease treatment.

2. The variant of Claim 1, wherein the insecticidally effective portion of said protoxin is an about 69 kD toxin.

3. The variant of Claim 1, wherein said variant is a BTS02618Aa protoxin or a BTS02618Ab protoxin.

4. The variant of Claim 2, wherein said toxin is a BTS02618Aa toxin or a BTS02618Ab toxin.

5. A DNA sequence encoding the variant of Claim 1 or a BTS02618Aa toxin or a BTS02618Ab toxin.

6. The DNA sequence according to Claim 5, the BTS02618Aa toxin is shown Seq ID. No. 8.

7. A chimeric gene comprising a DNA sequence encoding a BTS02618Aa toxin gene and a selectable marker gene.

8. The chimeric gene of Claim 7, wherein the selectable marker gene is the neo gene and the DNA sequence encoding said BTS02618Aa toxin gene is illustrated in Seq ID. No. 8.

9. The DNA sequence of SEQ ID. No. 6 encoding the BTS02618A toxin.

10. A BTS02618Aa toxin having an amino acid sequence as illustrated in SEQ ID. No. 9.

11. An insecticidal composition against Lepidopteran insects comprising an active ingredient selected from the group consisting of a more protease resistant BTS02618A protoxin, an insecticidally effective variant of a more protease resistant BTS02618A protoxin, a BTS02618Aa protoxin, an insecticidally effective BTS02618Aa protoxin portion, a BTS02618Aa toxin and variants thereof.

12. The insecticidal composition of Claim 12, wherein said Lepidopteran insects are Agrotis spp., Spodoptera spp., Plutella spp., Mamestra spp., Heliothis spp., Phthorimaea spp. and Ostrinia spp.

13. A transformed microorganism comprising a DNA sequence encoding a more protease resistant BTS02618A protein or a BTS02618Aa protoxin or BTS02618Aa toxin or insecticidally effective variants thereof.

14. The transformed mircroorganism according to Claim 13, wherein said microorganism is B. thuringiensis.

15. A transformed plant cell comprising a DNA sequence encoding a more protease resistant BTS02618A protein or an insecticidally effective bTS02618Aa gene part or a truncated bTS02618Aa gene or a bTS02618Aa chimeric gene or a hybrid thereof and a selectable marker gene.

16. The transformed plant according to Claim 15, wherein said plant is corn.

17. The transformed plant cell according to Claim 15 and Claim 16, wherein said selectable marker gene is the neo gene.

18. A plant or a seed thereof comprising a plurality of the plant cells of Claim 15 and Claim 16.

19. A plant genome containing, integrated therein one of the

DNA sequences according to any one of Claims 5 to 10.

20. A plant tissue, the cells of which have the plant genome of Claim 18.

21. A process for rendering a plant resistant to Lepidopteran insects comprising providing the plant with the plant genome of Claim 19.

22. The process according to Claim 21, wherein said Lepidopeteran insects are Noctuidae such as Spodoptera spp. and Agrotis spp., Pyralidae such as O. nubilalis, Gelechiidae such as Phthorimaea operculella and Yponomeutidae such as Plutella xylostella.

23. A process for producing plants and reproduction material of said plants including a heterologous genetic material stably integrated in the genome thereof and capable of being expressed therein in the form of a protein toxic to insects, comprising the non-biological steps of:

a) producing a transformed plant cell or a plant tissue including said heterologous genetic material starting from a plant cell or a plant tissue not expressing said protein;

b) producing a regenerated plant or reproduction material of said plant or both from said transformed plant cell or said plant tissue including said heterologous genetic material; and

c) biologically replicating said regenerated plant or reproduction material or both, wherein said step of producing said transformed plant cell or plant tissue including said heterologous genetic material comprises transforming said starting plant cell or plant tissue with a DNA sequence encoding a more protease resistant BTS02618A protein, a BTS02618Aa protoxin or insecticidally effective portions thereof and regulatory elements which enable the expression of said DNA sequence in said plant cell or plant tissue, allowing the stable integration of the gene part, or the truncated gene or the hybrid in transformed plant cell or plant tissue, as

well as in said plant and reproduction material produced therefrom throughout generations.

24. A process for controlling a Lepidopteran insect pest said process comprising the step of contacting the pest with a more protease resistant BTS02618A protoxin or insecticidally effective portions thereof or a BTS02618Aa protoxin or a BTS02618Aa toxin or the insecticidal composition of Claim 11.

25. The process according to Claim 24, wherein the Lepidopteran insects include Agrotis ipsilon, Agrotis segetum, Spodoptera exigua, Spodoptera littoralis, Spodoptera frugiperda, Mamestra brassica, Heliothis virescens, Ostrinia nubilalis, Phthorimaea operculella and Plutella xylostella.

26. An improved Bacillus thuringiensis crystal protein, in which at least one internal protease cleavage site has been inactivated by a deletion or substitution of at least one amino acid so that the insecticidal activity of said protein is substantially maintained upon protease cleavage and a DNA sequence encoding such a protein.

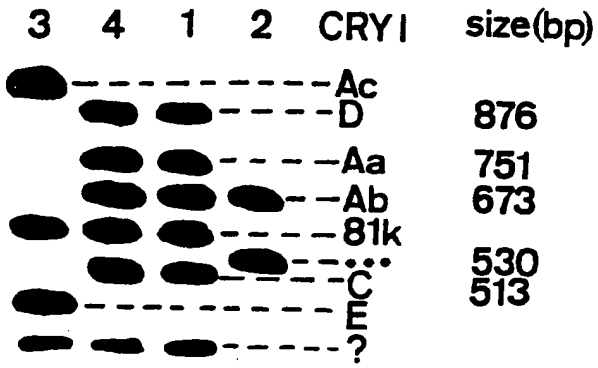
27. The improved Bacillus thuringiensis crystal protein according to Claim 26, wherein said protein is a BTS02618Aa protein or a BTS02618Ab protein.

28. A process for controlling Agrotis segetum or Phthorimaea operculella comprising the step of contacting Agrotis segetum or Phthorimaea operculella insect pests with a BTS02618A protoxin or BTS02618A toxin or variants thereof or a BTS02618Aa protoxin or a BTS02618Ab protoxin or insecticidally effective portions thereof.

29. A plant expressing a DNA sequence encoding the BTS02618Aa toxin of SEQ ID. No. 9 and a DNA sequence encoding the CryIAb toxin and/or a DNA sequence encoding the CryIB toxin.

30. The plant according to Claim 29, wherein said plant is corn.

Figure 1



INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 94/00553

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N1/20 C12N15/32 C12N5/04 C12N5/10 A01H5/00
A01H5/10 //(C12N1/20,C12R1:07)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 498 537 (MYCOGEN CORPORATION) 12 August 1992 see the whole document ---	1-30
A	WO,A,93 04587 (MYCOGEN CORPORATION) 18 March 1993 see the whole document ---	1-30
A	EP,A,0 358 557 (PLANT GENETIC SYSTEMS, N.V.) 14 March 1990 see the whole document ---	5-9,15, 17-23
A	WO,A,90 06999 (PLANT GENETIC SYSTEMS, N.V.) 28 June 1990 cited in the application see the whole document --- -/--	1-4, 10-14, 24,25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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'&' document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
21 June 1994	14. 07. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Ceder, O

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	FEBS LETTERS, vol.293, no.1,2, 18 November 1991, AMSTERDAM pages 25 - 28 SMULEVITCH ET AL. 'Nucleotide sequence of a novel ....' cited in the application see figure 2	5-9
A	EP,A,0 440 581 (CIBA-GEIGY AG) 7 August 1991 see abstract	1
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2

INTERNATIONAL SEARCH REPORT

Information on patent family members

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PCT/EP 94/00553

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